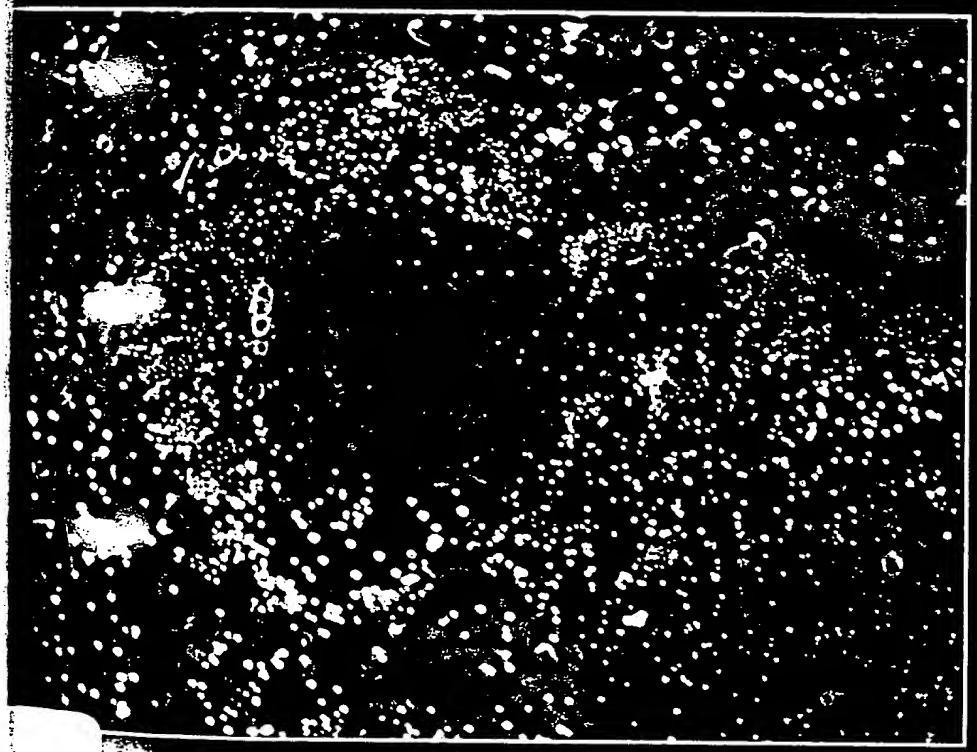
The Journal of Clinical Investigation



- molecular medicine / genetic disorders
- infection / inflammation / immunity
- hormones / cytokines / signaling
- cell growth & differentiation
- cellular, transport & organ physiology
- atherosclerosis / thrombosis / metabolism

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JCINAO 88(4) 1063–1434 (1991)

■ Cell Growth and Differentiation

■ Cellular, Transport and Organ Physiology

■ Atherosclerosis / Thrombosis / Metabolism

* Perspectives article
‡ Rapid Publication

Cover picture: Localization of urokinase-type plasminogen activator mRNA in tumor cells surrounding a horn pearl in a cutaneous squamous cell carcinoma. See related article in this issue by Sappino et al., pp. 1073–1079.

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■ Cell Growth and Diff rentiation

■ Cellular, Transport and Organ Physiology

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* Perspectives article
‡ Rapid Publication

Cover picture: Shown is a secondary structure model of the Na^+/glucose cotransporter of human intestinal microvilli, depicting a known N-linked complex of oligosaccharide and 12 postulated membrane spans. In two children of a Syrian family afflicted with glucose/galactose malabsorption a single missense mutation, aspartate (D) to asparagine (N) was found which destroyed transport activity and was shown to be the cause of this autosomal recessive disease. Prepared using CorelDraw2. See related article in this issue by Wright et al., pp. 1435–1440.

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Inhibition of Platelet Function by an Aspirin-insensitive Endothelial Cell ADPase

Thrombosis Regulation by Endothelial Cells

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Abstract

We previously reported that platelets become unresponsive to agonists when stimulated in combined suspension with aspirin-treated human umbilical vein endothelial cells. Inhibition occurred concomitant with metabolism of platelet-derived endoperoxides to prostacyclin by endothelial cells. We now demonstrate that if aspirin-treated platelets which fully respond to appropriate doses of agonists are exposed to aspirin-treated endothelial cells, they remain unresponsive despite absence of prostacyclin. Platelet inhibition is due in large part to ecto-ADPase activity on the endothelial cells. This was established by incubating aspirin-treated endothelial cells with ^{14}C-ADP. Radio–thin layer chromatography and aggregometry demonstrated that ^{14}C-ADP and induction of platelet activation decreased rapidly and concurrently. AMP accumulated transiently, was further metabolized to adenosine, and deaminated to inosine. The apparent K_m of the endothelial cell ADPase was 33–42 μM and the V_{max} 17–43 nmol/min per 10^6 cells, values in the range of antithrombotic potential.

Thus, at least three complementary systems in human endothelial cells control platelet responsiveness: a cell-associated, aspirin-insensitive ADPase which functions in parallel with fluid phase autacoids such as the aspirin-inhibitable eicosanoids, and the aspirin-insensitive endothelium-derived relaxing factor. (*J. Clin. Invest.* 1991.88:1690–1696.) Key words: nucleotidases • cell–cell interactions • platelet aggregation • platelet serotonin release • thrombosis

Introduction

Realization of the importance of vascular cell–cell interactions and transcellular metabolism has increased in recent years (1). This is particularly pertinent to the case of endothelial cells and platelets. Currently we hypothesize that endothelial cells control platelet reactivity via at least three mechanisms: a cell-associated ADPase system and two fluid phase reactants; eicosanoids such as prostacyclin (PGI_2)[1]; and the endothelium-derived relaxing factor (EDRF). In this report we extend previous studies on platelet inhibition by PGI_2 formed by aspirin-treated endothelial cells from platelet endoperoxides (2). Under experimental conditions in which EDRF was not measurable, we found that platelet reactivity was inhibited by endothelial cells even though both cell types were aspirin treated and PGI_2 was absent. Biochemical and functional data will be presented indicating that these aspirin-treated endothelial cells inhibit platelet function largely via a mechanism involving metabolism of ADP and consequent loss of its proaggregatory activity.

Methods

Preparation of platelet-rich plasma and platelet suspensions. Blood was collected from donors ~ 12 h after they had ingested 650 mg acetylsalicylic acid, aspirin (ASA). Platelet-rich plasma (PRP) was prepared using acid citrate-dextrose (citric acid, 38 mM; sodium citrate, 75 mM; glucose, 135 mM) as anticoagulant (3), with an initial whole blood centrifugation at 200 *g*, 15 min (25°C) and a second centrifugation of the PRP (90 *g*, 10 min) to eliminate most of the residual erythrocytes and leukocytes. PRP was maintained at room temperature under 5% CO_2-air.

Platelet suspensions, when used, were prepared from PRP as described previously (3). Final resuspension was in cold 0.15 M NaCl to a count of 1×10^8 platelets/20 μl. The suspension was kept in a closed container at 4°C.

Preparation of endothelial cell suspensions. Cultured human endothelial cells (P2-P8) derived from umbilical cords (4) were treated with 1 mM ASA (10 μl of 1 M ASA in ethanol/10 ml) for 30 min at 37°C (2). Cells were washed in Hepes-buffered saline and detached with collagenase-EDTA-BSA solution (4). An equal volume of human serum–containing medium was added, the cells centrifuged at 500 *g* for 10 min (22°C), and finally resuspended in ASA-free buffer (0.25 ml/T-75 flask). Resuspension buffer was either Tris-Saline-Glucose (TSG) (Tris, 15 mM; NaCl, 134 mM; glucose, 5 mM, pH 7.4) or Hepes, 5 mM; NaCl, 140 mM; KCl, 5 mM; $CaCl_2$, 1.29 mM; $MgCl_2$, 1.20 mM, pH 7.45. Indomethacin was then added to a concentration of 10 μM. Suspensions were generally maintained at room temperature or at 4°C as specified. Endothelial cell (EC) counts averaged 4,466 cells/μl.

Aggregation studies with combined suspensions of ASA-treated platelets and ASA-treated EC. These experiments were carried out similarly to those previously reported in which ASA-treated EC, but untreated platelets were studied (2). ASA-PRP containing 1×10^8 platelets or ASA-treated washed platelets (1×10^8) in buffer were preincubated (3 min, 37°C) in siliconized cuvettes containing stirring bars in an aggregometer. When used, SOD or other substances of interest such as hemoglobin, $FeSO_4$, or nitroprusside were included with the platelets or during preincubation. ASA-treated EC were then added, followed in 1 min by the agonist. In the case of TSG buffer, which contained no calcium, Ca^{++} (3 mM) was included 15 s before thrombin or collagen. Total volumes were adjusted to 500 μl with buffer. The aggregation response was recorded over a 5-min period in a Lumiaggregometer (Chrono-Log Corp., Havertown, PA). Control "platelet-poor" cuvettes contained equal numbers of EC to those in "platelet-rich" cuvettes in order to correct for light absorption by the nonaggregating EC.

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1. *Abbreviations used in this paper:* ASA, acetylsalicylic acid, aspirin; EC, endothelial cell(s); EDRF, endothelium-derived relaxing factor; 5-HT, serotonin, 5-hydroxytryptamine; NO, nitric oxide; PGI_2, prostacyclin; PRP, platelet-rich plasma.

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Studies of possible inhibitory properties of ASA-EC supernatants. EC were tested for their inhibitory activity as above, using ADP, thrombin, or collagen as platelet agonist. Separate aliquots of EC were equilibrated at 37°C in polypropylene microfuge tubes with stirring (in the presence or absence of SOD, 60 U/ml). They were then incubated (15 s) with specific EC stimuli such as bradykinin (100 nM), acetylcholine (2 μM), histamine (10 μM), or thrombin (1.25 U/ml), followed by rapid centrifugation (10 s, 15,600 *g*; Eppendorf Inc., Fremont, CA). Supernatants were transferred to aggregometer cuvettes containing 1 × 10^8 ASA-treated platelets (either washed or in PRP). Platelet agonists (ADP, collagen, or thrombin; see below) were added 15–20 s later. Total volumes in the cuvettes were adjusted to 500 μl with buffer. Controls were carried out to evaluate effects of carry-over of EC agonists on platelet aggregation. In the case of thrombin, the quantity carried over served as platelet agonist in the cuvette.

Time course of EC ADPase activity. ASA-EC in a total volume of 400 μl, plus 15 μM (^{14}C) ADP (41.7 mCi/mmol; New England Nuclear, Boston, MA), or buffer plus (^{14}C) ADP for controls, were incubated as in the other centrifugation studies for varying periods of time (15 s–30 min). Stirring bars were rapidly removed and the tubes centrifuged as above. A 100-μl aliquot of supernatant was transferred to the cuvette containing platelets and the aggregation response recorded. In control experiments containing no EC, the final ADP concentration in the cuvettes was 3 μM.

Immediately after removal of the initial 100 μl of supernatant, an additional 200 μl was placed in a polypropylene microfuge tube containing 10 μl of "stop solution" (5). The stop solution stock consisted of 160 mM disodium EDTA, neutralized to pH 7, plus 17 mM ADP in ice-cold physiological saline. The stop solution was added to prevent any possible further degradation of ADP. Tubes were then vortexed and kept on ice until the experiment was concluded. Scintillation counting was performed on 5 μl from each sample. Tubes were stored at −70°C for thin-layer chromatography of ADP and its metabolites.

Incubations could also be carried out with endothelial cell monolayers in multiwell plates (well capacity 1.5 ml, growth area 2 cm^2; 3847; Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ), without stirring, using the same ADP concentration and total volume. Plates were secured by weights in a 37°C water bath. At indicated time intervals, supernatants were aspirated and centrifuged as above.

The well system was also used to determine the ADPase activity of fresh endothelial cells. Cells derived from two cords were pooled and divided between nine wells. Within 1–2 h of incubation, the endothelial cells were adherent. This permitted assay of EC ADPase activity free from contaminating erythrocytes, which were discarded in the supernatant.

Aggregation studies using supernatants of either thrombin-stimulated platelets or thrombin-stimulated platelet–endothelial cell mixtures as platelet agonist. Washed platelets (1 × 10^8) from a donor who had ingested aspirin, or platelets plus 2 × 10^6 ASA-treated endothelial cells were preincubated for 3 min and then stimulated with thrombin (1 U/ml), in the presence of 3 mM Ca^{++} and 20 μM hemoglobin. Total incubation volume was 400 μl. The microfuge tubes were incubated for 5 min after stimulation, stirring bars removed and centrifugation carried out for 10 s (Eppendorf). A control tube containing thrombin but no cells was also incubated for 5 min.

100 μl of supernatant was rapidly transferred to an aggregometer cuvette containing 227 μl of PRP from the same donor (1.3 × 10^8 platelets) and buffer (total volume of the assay cuvette was 500 μl). The aggregation response was recorded for 4 min.

TLC studies of nucleotides, nucleosides, and bases. TLC was carried out on fluorescent silica gel 60 F_{254}-coated plastic sheets (20 × 20 cm; EM Separations, Gibbstown, NJ). 15 μl of radioactive sample was applied and dried under a stream of air. At each point of application, 2 μl of a mixture of standards was added and dried. The standard mixture consisted of 2 mg each of the following compounds: ATP, ADP, AMP, inosine, hypoxanthine, adenosine, and adenine in distilled water (total vol = 1 ml) (6). The solvent system for nucleotides, nucleosides, and bases consisted of isobutyl alcohol/1-pentanol/ethylene glycol monoethyl ether/NH_4OH/H_2O (90:60:180:90:120) (solvent 1) (6). For separation of hypoxanthine and adenosine, the solvent system consisted of 1-butanol/ethyl acetate/methanol/NH_4OH (7:4:3:4) (solvent 2) (7).

Solvent systems were prepared at least 48 h before use and added to the tank 1 h before insertion of plates (8). Development of plates was carried out for 5 h, 10 min in solvent 1, or 4 h in solvent 2. Plates were dried under a stream of warm air, the separated compounds visualized under ultraviolet light (254 nm), and scanned for radioactivity with an RTLC multi-scanner (Radiomatic Instruments & Chemical Co., Inc., Tampa, FL).

Substrate concentration curve of EC ADPase. Aspirin-treated EC (72,233/400 μl total vol) in TSG buffer were incubated with stirring for 5 min (37°C) with 1.2–80.1 μM ADP containing 2.4 μCi of (^{3}H) ADP (trisodium salt, 27.3 Ci/mmol; New England Nuclear), in the presence of 10 μM dipyridamole. The latter was used to prevent reuptake of adenosine and consequent resynthesis of ADP (7, 9). Ca^{++} (1.22 mM) was added 30 s before ADP. As in the time course experiments described above, tubes were then centrifuged, supernatants treated with stop solution, aliquots counted for total radioactivity, and TLC performed. The dipyridamole stock solution was prepared in glycine-HCl buffer (0.05 M, pH 2.8). During these experiments, EC stock suspensions were stored at 4°C.

(^{14}C) Serotonin, 5-hydroxytryptamine (5-HT) release. For platelet labeling, 0.2 nmol (^{14}C) 5-HT creatinine sulfate (54 mCi/mmol; Amersham Corp., Arlington Heights, IL) was added directly to the anticoagulant for each ml of anticoagulated blood. (^{14}C) 5-HT uptake was determined 1 h after blood collection by comparison of radioactivity in 50-μl aliquots of PRP and platelet-poor plasma. Assays of the effects of EC on platelet (^{14}C) 5-HT release and aggregation were carried out in cuvettes in the usual manner, using 1 × 10^8 labeled platelets in suspension or PRP. To prevent reuptake of released 5-HT, imipramine (2.5 μM) was added 90 s before the agonist. Controls containing labeled platelets without agonist were carried out to measure any release of (^{14}C) 5-HT attributable to stirring alone. Reactions were stopped 4 min after stimulation by placing the cuvettes on ice. Cuvette contents were transferred to microfuge tubes and centrifuged for 3 min (10,000 *g*, 4°C). 50-μl aliquots of each supernatant were counted in 4 ml Aquasol-2 and compared to total platelet counts.

Additional methods. Treatment of platelets with methylene blue, an inhibitor of soluble guanylate cyclase, was essentially according to Alheid et al. (10). Methylene blue was added to a washed platelet suspension at a concentration of 10 μM. The suspension was then left at room temperature for 30 min, centrifuged at 1,450 *g* (15 min, 4°C) and the pellet resuspended in cold 0.15 M NaCl.

Oxyhemoglobin was prepared from bovine hemoglobin (type 1; Sigma Chemical Co., St. Louis, MO) by the method of Martin et al. (11). Human hemoglobin yielded identical results. Briefly, a 1-mM solution of the commercial mixture of oxyhemoglobin and methemoglobin was reduced with a 10-fold molar excess of sodium dithionite, which was then removed by dialysis. Purity was checked spectrophotometrically and aliquots frozen at −70°C.

Adenosine-5′-O-(2-thiodiphosphate) trilithium salt (ADP-β-S) was obtained from Boehringer Mannheim, Indianapolis, IN.

Prostacyclin production was measured by RIA for 6-keto-$PGF_{1\alpha}$ (DuPont-New England Nuclear).

Results

Endothelial cells inhibit platelet reactivity in totally aspirin-treated systems. When ASA-treated washed platelets were stimulated by agonists in the presence of ASA-treated endothelial cells, platelet aggregation was inhibited (Fig. 1). This occurred under conditions where control ASA-platelets alone were fully aggregated by the same quantity of stimulus (Fig. 1). RIA measurements verified that no PGI_2 had formed in these experi-

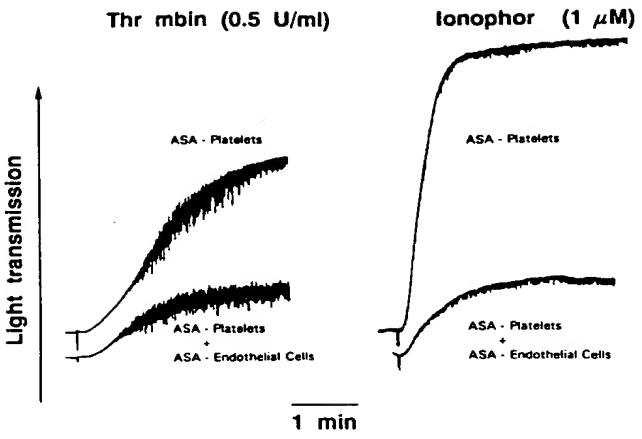

Figure 1. Inhibition of platelet aggregation by endothelial cell suspensions. The upper curves (controls) represent the response of washed, aspirin-treated platelets to thrombin and ionophore, respectively. The lower curves depict inhibited platelet responsiveness when aspirin-treated endothelial cells were present. Aspirin treatment of both cell types prevented transcellular metabolism of platelet endoperoxide to prostacyclin by the endothelial cells (2).

ments. As shown in Fig. 2 *A*, PRP from a donor who had ingested aspirin was fully aggregated by 1 μM ADP. In contrast, when this PRP was stimulated in the presence of 1×10^6 ASA-EC, the inhibited aggregation curve was characterized by a brief ascending limb followed by reversal. The pattern of reversibility was reminiscent of previous experiments in this laboratory wherein ADP released from platelets by PGH_2 was intentionally removed by enzymatic means (apyrase) (Fig. 2 *B*).

Concurrent metabolism of ADP by endothelial cells and loss of its potential as a platelet agonist. The hypothesis that an ADPase activity was present on these human endothelial cells and could possibly account for their platelet-inhibitory properties was tested biochemically and functionally. (^{14}C) ADP (15 μM) was incubated with ASA-EC for increasing periods of time. The supernatants were then examined for their content of residual (^{14}C) ADP and its metabolites, as well as for the platelet aggregating potential of the unmetabolized (^{14}C) ADP. Results are depicted in Figs. 3 and 4. When compared to (^{14}C) ADP controls which had been incubated with buffer alone, the presence of EC resulted in a progressive decrease in (^{14}C) ADP concentration. This was paralleled by loss of supernatant proaggregatory activity as measured by the decrease in maximum height of the platelet aggregation curves. Comparable results were obtained whether endothelial cell suspensions (Fig. 3) or monolayers (Fig. 3, *inset*) were employed. ADPase activity was also present on freshly adherent, but uncultured endo-

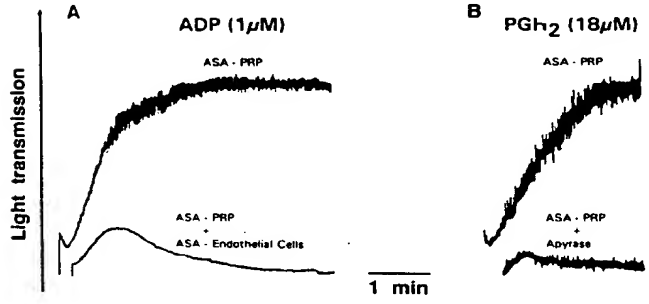

Figure 2. Comparison of the inhibition of ADP-induced aggregation in PRP by endothelial cells (*A*), with that due to enzymatic removal by apyrase of platelet ADP released by prostaglandin endoperoxide (PGH_2) stimulation (*B*). Similarities in the shapes of the curves of inhibited aggregation (lower curves) suggest enzymatic removal of ADP in *A* as well.

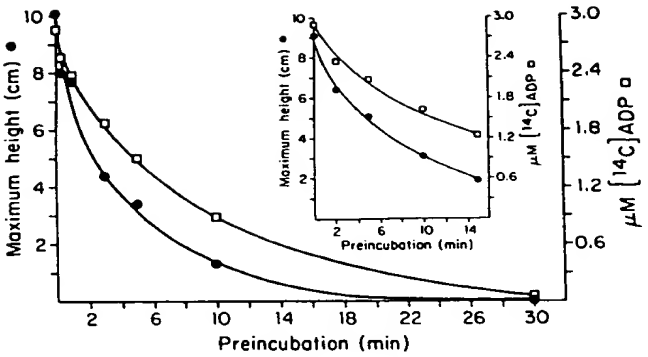

Figure 3. Time course of metabolism of (^{14}C) ADP by aspirin-treated endothelial cell suspensions. The decrease in (^{14}C) ADP concentration as measured by thin-layer radiochromatography (— □ —), was accompanied by loss of platelet proaggregatory activity (— ● —) of supernatants derived from incubation of endothelial cells with (^{14}C) ADP. This also occurred when endothelial cell monolayers were used (*inset*). Results shown are from representative experiments in which 72,215 suspended EC or 84,625 EC in monolayers were assayed.

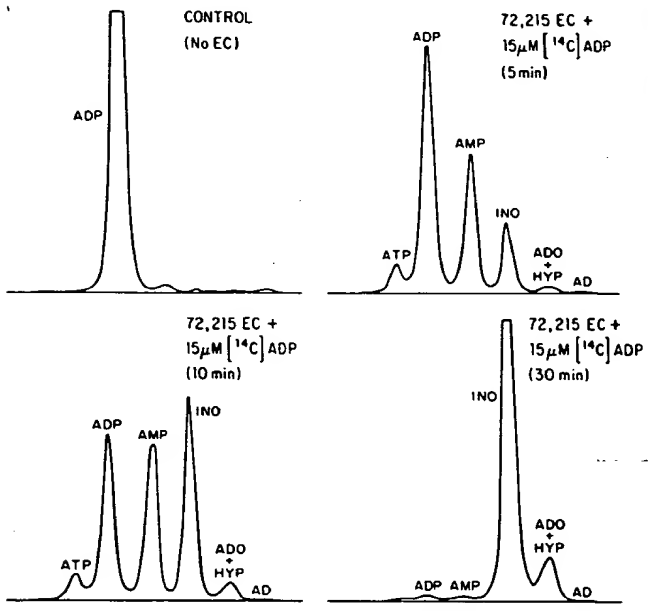

Figure 4. Time course of formation of metabolites of (^{14}C) ADP by aspirin-treated endothelial cell suspensions. Cell-free supernatants derived from incubations of endothelial cells with (^{14}C) ADP were analyzed for (^{14}C) ADP and its metabolites by TLC.

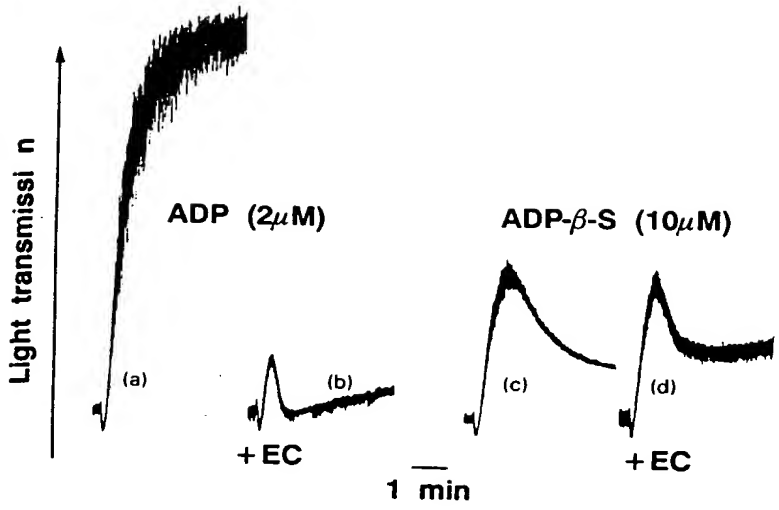

Figure 5. Studies with ADP-β-S, a nonmetabolizable ADP analogue that activates platelets. (*a*) ADP elicited a full aggregation response in aspirin-treated platelets. (*b*) Inhibition of the ADP response by aspirin-treated endothelial cells. (*c*) Aggregation response of aspirin-treated platelets to ADP-β-S. (*d*) Aggregation induced by ADP-β-S was not appreciably influenced by the presence of endothelial cells, which were unable to metabolize the ADP-β-S.

thelial cells. There was only a slight decrease in ADPase activity concomitant with cell passage. Fig. 4 depicts radio-TLC scans of the metabolites of (^{14}C) ADP after 5, 10, and 30 min incubation with ASA-EC suspensions. At the 5-min time point, ADP had decreased to 51% of total nucleotides, nucleosides, and bases. In addition, AMP (28%) and inosine (13%) were identified, together with trace quantities of adenosine, hypoxanthine, and adenine. By 10 min, concentrations of ADP, AMP, and inosine averaged 30%, 29%, and 29%, respectively. Inosine (85%) was the major (^{14}C) ADP metabolite at the 30-min interval, and ADP itself was virtually absent. Chromatography with solvent system 2 indicated that hypoxanthine (8%) had been synthesized. As can be seen in Fig. 3, the absence of ADP correlated with total loss of aggregatory activity of the ASA-EC supernatant.

The presence or absence of endothelial cells did not affect the recovery of total radioactivity in supernatants. This indicated that nonspecific adsorption of ADP to the endothelial cells was not the cause of the decrease in supernatant proaggregatory activity.

Endothelial cell inhibition of ADP-induced platelet reactivity requires ADP hydrolysis. Experiments were carried out with ADP-β-S, a structural analogue of ADP that activates platelets, but is not metabolized by ADPases (12). As shown in Fig. 5, ASA-platelets stimulated alone with 2 μM ADP demonstrated a full aggregation response (*a*). In the presence of 1×10^6 ASA-EC, this response was markedly attenuated and reversed (*b*). ADP-β-S (10 μM) induced a reversible aggregation response of lesser amplitude than ADP (2 μM) with ASA-platelets alone (*c*). However, in contrast to results with ADP, ASA-EC did not exert an appreciable inhibitory effect on ADP-β-S-stimulated ASA-platelets (*d*). These results provided further evidence for ADP hydrolysis by EC as a major mechanism underlying their inhibitory effect on stimulated platelets.

Kinetic parameters of human endothelial cell ADPase. We measured the rate of hydrolysis of (^{3}H) ADP by ASA-EC as a function of substrate concentration. Apparent K_m and V_{max} values were determined from Lineweaver-Burk plots of the data (Fig. 6). In two experiments, the K_m ranged from 33 to 42 μM and the V_{max} from 17 to 43 nmol/min per 10^6 cells. These

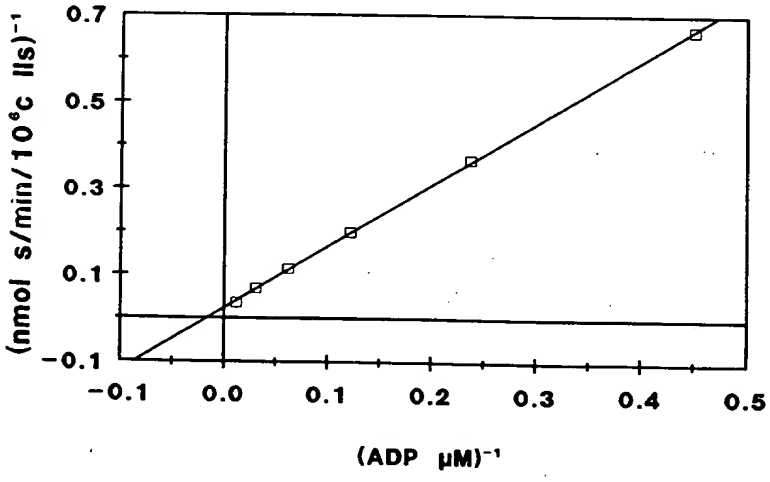

Figure 6. Lineweaver-Burk plot relating the rate of (^{14}C) ADP hydrolysis by aspirin-treated endothelial cells to ADP concentration. The K_m calculated from these data was 33 μM and the V_{max} was 42.5 nmol/min per 10^6 cells. Results depicted are representative of two separate experiments.

kinetic parameters are compared in Table I with values reported in the literature for porcine aortic endothelial cells (13, 14).

Endothelial cell ADPase inhibits platelet reactivity independent of EDRF. ASA-endothelial cells (which did not produce PGI_2), inhibited platelet aggregation by an aspirin-insensitive mechanism. This inhibition could have been due in part to a fluid phase reactant such as EDRF in combination with the EC ADPase activity described above. To test this possibility, known modulators of EDRF activity were added to the platelet-EC incubations. Inhibition of platelet aggregation by endothelial cells was not affected by: (*a*) hemoglobin or ferrous ion, which inactivate EDRF/nitric oxide (NO); (*b*) methylene blue, which prevents the effects of EDRF/NO by inhibiting soluble guanylate cyclase; or (*c*) superoxide dismutase, a potentiator of EDRF/NO via removal of superoxide radicals.

Supernatants of EC that had been previously stimulated (15 s) with bradykinin, acetylcholine, or histamine were rapidly transferred to cuvettes containing platelets. There was little (< 10%) if any inhibition of ADP, thrombin, or collagen-stimulated platelet aggregation. This indicated that the platelet inhibitory activity observed in the presence of ASA-endothelial cells was not transferable, and therefore was mainly surface associated. When endothelial cells were prestimulated with thrombin, the quantity present in the transferred supernatant served as agonist in the aggregometry cuvette. Since the same degree of aggregation was obtained with this supernatant (even after a 3-min incubation of thrombin with EC) as with thrombin alone, nonspecific adsorption of agonist to the EC could again be ruled out as the possible etiology of platelet inhibition.

Release of (^{14}C) 5-HT from stimulated platelets was used as another parameter to gauge the effects of ASA-endothelial cells on platelet reactivity. ASA-endothelial cells inhibited 5-HT release from activated platelets as well as their aggregation (Table II). Hemoglobin did not reverse the inhibitory effect of the ASA-endothelial cells on either 5-HT release or aggregation.

Endothelial cells reverse the capacity of thrombin-elicited platelet releasates to enhance platelet aggregation. To more closely relate the loss of thrombin-induced proaggregatory activity to EC ADPase action, the following experiments were performed. Supernatants from platelets that had been stimulated with thrombin (1 U/ml) in the absence or presence of endothelial cells were used as agonists for platelet aggregation. Curve (*a*) in Fig. 7 was generated by the threshold level of thrombin (0.2 U/ml) transferred from the incubation tube to the aggregometry assay cuvette. The enhanced aggregation response in curve (*c*) was evoked by the combined presence of transferred thrombin-induced platelet releasate in addition to transferred thrombin in the supernatant. Comparable synergy was demonstrated in control experiments in which thrombin (0.2 U/ml) plus concentrations of ADP expected to be released during the initial incubation were added directly to the aggregometer cuvette (data not shown). The presumed concentration of ADP released into the incubation tube, calculated from values reported in the literature (15) was 6.25 μM. The kinetic parameters we derived for the human endothelial cell ADPase under study indicate that this quantity of ADP would have been hydrolyzed by the 2×10^6 EC present during our 5-min incubation. It can be seen in curve (*b*) Fig. 7 that the presence of EC did indeed reverse the enhanced aggregation.

Table I. Kinetic Parameters for Endothelial Cell ADPases from Different Sources

	K_m	V_{max}	V_{max}/K_m
	μM	*nmol/min per 10^6 cells*	
Human umbilical vein endothelial cells	38	30	0.789
Porcine aortic endothelial cells (13)	155	9.2	0.059
Porcine aortic endothelial cells (14)	247	6.2	0.025

Aspirin-treated endothelial cells were incubated with (^{3}H) ADP. After 5 min the tubes were centrifuged and thin-layer chromatography performed on the supernatants. The rate of hydrolysis of ADP was measured as a function of substrate concentration. Apparent K_m and V_{max} values were determined from Lineweaver-Burk plots as shown in Fig. 6. Values for human umbilical vein endothelial cells represent averages from two separate experiments. These parameters are compared with values reported in the literature.

Table II. Inhibition of Serotonin Release from Stimulated Aspirin-treated Platelets by Aspirin-treated Endothelial cells

	Serotonin release (%)	
	Collagen	Thrombin
	10 μg/ml	*0.3 U/ml*
Platelets alone	68	66
+ 1 × 10^6 endothelial cells	26	0
+ 1 × 10^6 endothelial cells + hemoglobin (20 μM)	15	4
+ 0.5 × 10^6 endothelial cells	48	—
+ 0.5 × 10^6 endothelial cells + hemoglobin (20 μM)	31	—

Values represent radioactive serotonin released into the supernatant, as percent of total radioactive serotonin in the labeled platelets. Hemoglobin was used as an inhibitor of EDRF/NO activity. If the inhibition of serotonin release were due to EDRF/NO, the inhibition of release would have been reversed in the presence of hemoglobin. These results are representative of four separate experiments.

Discussion

In our earlier studies of platelet responsiveness to agonists when in combined suspension with human endothelial cells, we correlated the observed inhibition of platelet aggregation with prostacyclin formation (2). Under those experimental conditions, the endothelial cells had been pretreated with aspirin, but the platelets were not. Prostacyclin formation was demonstrated to result from metabolism of platelet-derived endoperoxides by endothelial cells in apposition. In the last decade, at least two endothelial cell-associated platelet inhibitory substances in addition to PGI_2 have been identified. These are endothelial cell ecto-ADPases (9, 16) and the endothelium-derived relaxing factor (EDRF/NO) (17–20). Production of EDRF and the action of endothelial cell ADPases are insensitive to aspirin and could have contributed at least in part to results observed in earlier studies (2).

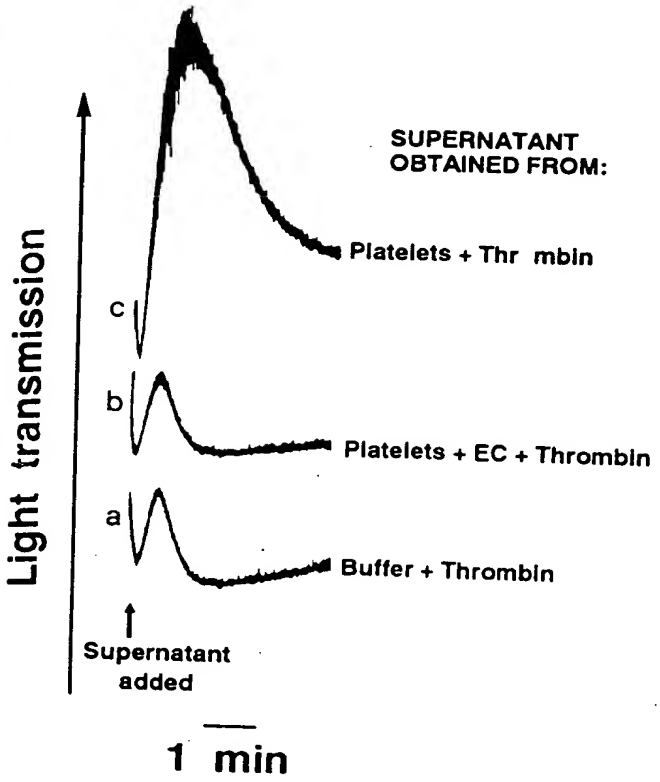

Figure 7. Aggregation response of platelets in PRP to supernatants derived from thrombin-stimulated platelets or thrombin-stimulated platelet–endothelial cell mixtures. (*a*) Aggregation due to thrombin carried over in the supernatant of a buffer control which contained no cells. In (*c*) the presence of thrombin-induced platelet releasate in the supernatant produced an enhanced aggregation response. The enhancement of supernatant aggregatory potency as shown in (*c*) was reversed by the interaction of endothelial cell ADPase with the platelet releasate as shown in (*b*).

We therefore performed experiments using both aspirin-treated platelets and aspirin-treated endothelial cells, thereby eliminating production of any cyclooxygenase-derived eicosanoids. As shown in Figs. 1 and 2 and Table II, endothelial cells inhibited platelet responsiveness to all agonists tested even when the entire system was aspirin-treated. The endothelial cell-induced inhibition was cell associated since the inhibitory activity was not present in supernatants from endothelial cells even when they had been stimulated with known agonists for EDRF/NO. Substances known to reverse or enhance EDRF/NO activity had no influence on the inhibitory effects of endothelial cells on the platelet responsiveness observed in these experiments. This is shown for hemoglobin in Table II.

When ADP-induced platelet aggregation was reversed in the presence of endothelial cells, the shape of the recorded pattern of reversal suggested that ADP had been enzymatically removed (Fig. 2). In this regard, it was possible to biochemically and functionally correlate metabolism of ADP by ASA-EC with disappearance of its properties as an agonist for platelet aggregation (Figs. 3 and 4). The fact that similar activity was demonstrable with either endothelial cell suspensions or adherent monolayers suggests that the ADPase activity is located on the luminal surface of the vessel and would interface directly with platelets. Comparable results have been reported by Crutchley and associates with bovine pulmonary arterial endothelial cells (7) and Glasgow et al. with human endothelial cell monolayers (21).

As shown in Table I, values obtained with our human endothelial cell preparations for the apparent K_m and catalytic efficiency (V_{max}/K_m) with ADP as substrate, compared favorably with those reported for porcine aortic endothelial cells (13, 14). Calculations using these figures indicate that the quantity of ADP hydrolyzable by our endothelial cell suspensions would be sufficient to result in inhibition of platelet aggregation. For example, 0.5×10^6 EC would metabolize 2 μM ADP to 0.8 μM in 30 s.

In the case of agonists other than ADP, such as thrombin, collagen, and ionophore, hydrolysis of released platelet ADP may also be involved in the loss of platelet responsiveness (Fig. 1, Table II). Calculations based on results depicted in Fig. 7 indicated that enhancement of threshold thrombin aggregation was attributable to thrombin-released platelet ADP. Removal of this ADP by endothelial ecto-ADPase reversed the enhancement, and aggregation reverted to the original threshold level. This occurred in the total absence of endothelial cell PGI_2 and EDRF and emphasizes the role of endothelial cell ADPases in control of platelet recruitment.

A major reason for elucidating biochemical and functional properties of endothelial cell–associated ecto-ADPases is that they can exert a significant effect on platelet reactivity independent of the action of other known endothelium derived inhibitors. For evaluation of the entire known spectrum of EC control of platelet reactivity ("thromboregulation"), cell preparations which simultaneously produce PGI_2, EDRF/NO, and possess ADPase activity will require additional assessment. It is also possible that as yet undefined endothelial cell thromboregulators could have contributed to results obtained by ourselves and others.

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